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foxD5a, a *Xenopus* Winged Helix Gene, Maintains an Immature Neural Ectoderm via Transcriptional Repression That Is Dependent on the C-Terminal Domain

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***Xenopus foxD5a*, the full-length *fork head* gene previously described as a PCR fragment (XFLIP), is first detectable at stage II of oogenesis. Low-abundance maternal transcripts are localized to the animal hemisphere of the cleavage embryo, and protein can be translocated to the nucleus prior to the onset of zygotic transcription. Zygotic expression is strongest in the presumptive neural ectoderm at gastrula and neural plate stages, but there is minor paraxial mesodermal expression during primary gastrulation that becomes significant in the tail bud during secondary gastrulation. Expression of *foxD5a* in animal cap explants induces elongation and expression of mesodermal, neural-inducing, and early neural-specifying genes, indicating a role in dorsal axis formation. Zygotic *foxD5a* expression is induced strongly by siamois, moderately by cerberus, weakly by Wnt8 and noggin, and not by chordin in animal cap explants. Expression of *foxD5a* in whole embryos has differential dorsal and ventral effects. Ventral mRNA injection induces partial secondary axes composed of expanded mesodermal and epidermal tissues, but does not induce ectopic neural tissues. Dorsal mRNA injection causes hypertrophy of the neural plate and expansion of early neural genes (*sox3* and *otx2*), but this is not the result of increased proliferation or expanded neural-inducing mesoderm. The neural plate appears to be maintained in an immature state because *otx2* expression is expanded and expression of *en2*, *Krox20*, proneural genes (*Xnrgn1*, *neuroD*) and a neural differentiation gene (*n-tubulin*) is repressed in *foxD5a*-expressing cells. These results indicate that *foxD5a* maintains an undifferentiated neural ectoderm after neural induction. Expression of *foxD5a* constructs fused with the *engrailed* repressor domain or with the VP16 activation domain demonstrates that FoxD5a acts as a transcriptional repressor in axis formation and neural plate expansion. Deletion constructs indicate that this activity requires the C-terminal domain of the protein.**

Key Words: **Organizer; Nieuwkoop Center; XFD-12′; winged helix; neural induction; tail bud.**

INTRODUCTION

Cell fate determination and differentiation result from the activation of cascades of genes in a functional hierarchy. In the early development of *Xenopus*, zygotic gene activation occurs at the midblastula transition (MBT) (Newport and Kirschner, 1982) after a period of molecular prepatterning and cell division driven by maternal factors (reviewed in Sullivan *et al.*, 1999). After zygotic activation, signaling centers such as the Nieuwkoop Center (Nieuwkoop, 1973) and the Organizer (Spemann and Mangold, 1924) impart a dorsal character to mesoderm and the overlying ectoderm. Transcription factors that may be involved in the pattern-specifying functions of these signaling centers are of particular interest since elucidation of the components that regulate them is well under way (Harland and Gerhart, 1997; Heasman, 1997; Moon and Kimelman, 1998; Kessler, 1999).

An important class of transcription factors with diverse roles in developmental processes is the *fork head* gene family. Constituent genes contain a highly conserved

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~110-residue DNA-binding domain, also named the winged helix domain (WHD) for its butterfly-like tertiary structure when bound to DNA (Clark *et al.,* 1993). The WHD was first identified in the product of the *Drosophila fork head* gene, which is required for proper development of embryonic anterior and posterior termini (Weigel *et al.,* 1989), and in the rat hepatocyte nuclear factor HNF3*β*, which is required for notochord formation (Lai *et al.,* 1991; Ang and Rossant, 1994). Well over 100 members of the *fork head* family have been identified in species spanning the phylogenetic spectrum from fungi to primates (reviewed in Kaufmann and Knöchel, 1996). The expression of *fork head* genes often is tissue-specific and developmentally regulated, and some members regulate tissue competence (Vermaak *et al.,* 1998; Zaret, 1999). Sequence variations within the WHD have been used to classify the Fork head proteins (Kaufmann and Knöchel, 1996; Lef *et al.,* 1996) and have been ascribed a role in selective DNA binding (Kaufmann *et al.,* 1995; Marsden *et al.,* 1997). In addition, the gene regulatory function of several Fork head proteins resides in the highly divergent regions outside the WHD (Pani *et al.,* 1992; Qian and Costa, 1995; Chang *et al.,* 1996; Freyaldenhoven *et al.,* 1997b). A recently revised system for naming members of the *fork head* family (Kaestner *et al.,* 2000) has been devised, renaming this family the *fox* (*fork head box*) genes.

Several *fox* genes have been characterized in *Xenopus* (reviewed in Knöchel and Kaufmann, 1997). Notable among these from the standpoint of early development are *XFKH-1* (= *XFD-1′*, *pintallavis* = *XFD-1*, new nomenclature = FoxA4a) and *XFD-6* (FoxD3a), both of which are expressed predominantly in the dorsal lip of the blastopore (i.e., the Organizer) during gastrula stages (Dirksen and Jamrich, 1992, 1995; Knöchel *et al.,* 1992; Ruiz i Altaba and Jessell, 1992; Scheucher *et al.,* 1995). A PCR fragment of another *fox* gene, called XFLIP because of its expression in the dorsal blastopore lip, was previously described based on RT-PCR data (King and Moore, 1994). We cloned its full-length cDNA (Sullivan and Moody, 1998), which is renamed *Xenopus foxD5a* in accordance with the new nomenclature. A recent report presented the amino acid sequences of FoxD5a (= XFD-12) and two of its alleles (XFD-12′/12″), along with aspects of the expression pattern of the *XFD-12′* allele (Sölter *et al.,* 1999). In this study we extend the characterization of *foxD5a* expression by showing that *foxD5a* is synthesized as early as stage II of oogenesis and that maternal transcripts become localized to the animal half of the cleaving embryo, the precursor field for dorsal axial mesoderm and neural ectoderm. Injected *foxD5a* mRNA causes the elongation of animal cap explants by upregulating mesodermal, neural-inducing, and neural-specifying plate genes. In turn, *foxD5a* expression is induced strongly by siamois, moderately by cerberus, weakly by Wnt8 and noggin, and not by chordin. In whole embryos, *foxD5a* does not induce ectopic neural ectoderm, but expands the native neural ectoderm independently of proliferation or expanded underlying mesoderm. The concomitant expansion of anterior markers and early neural-specifying genes and repression of more posterior and proneural genes suggest that *foxD5a* functions as an intermediary between neural induction and neural differentiation, maintaining the neural ectoderm in an immature state. We provide evidence that these activities are likely the result of transcriptional repression and require domains C-terminal to the WHD.

MATERIALS AND METHODS

Cloning and Sequence Analysis

A mouse BF2 probe (Hatini *et al.,* 1994) was used to screen a cDNA library derived from *Xenopus* animal caps that had been anteriorized with ammonium chloride (Mathers *et al.,* 1997). Three rounds of screening yielded two plaques that contained inserts of >1.5 kb. These were cloned into pBluescript SK$^-$ vectors and subjected to PCR using primers encoding conserved WHD sequences KPPYSY (5′-AAGCCTCCTTACTCGTAC-3′) and EPGNPG (5′-TCCTGGATTTCCTGGTTC-3′), with subsequent sequencing of the amplicons to verify the clones as members of the *fork head* family. Comparison of the amplicon DNA sequences to GenBank showed that they contained within them a region that exactly matched XFLIP, a 95-bp amplicon described previously (King and Moore, 1994). Two clones were then sequenced completely in both directions using nested primers. The larger of the two clones (*Xenopus foxD5a*) contained a lengthy potential open reading frame and flanking untranslated regions. Sequence comparisons to GenBank were performed using PSI-BLAST (Altschul *et al.,* 1997) and retrieved sequences were aligned using CLUSTAL W (Thompson *et al.,* 1994) with subsequent manual adjustment. Potential translational start sites were ranked using Netstart 1.0 (Pedersen and Nielsen, 1997). The complete *foxD5a* cDNA sequence was deposited in GenBank under Accession No. AF162782.

Expression Constructs and Microinjection

In all cases PCR amplification was performed using high-fidelity polymerases [Clontech (Palo Alto, CA) or Life Technologies, Inc., Rockville, MD], and all fusions and deletion constructs were verified by sequencing. The longest *foxD5a* open reading frame (MSFQ. . .PGCY, 352 aa) was amplified using primers with added restriction sites (*Stu*I/XfoxD5a85F: 5′-GCATAGGCCTATGAGCTTTAGCCAGGAGTCTGG-3′; *Xba*I/XfoxD5a1140R: 5′-ATCGTCTAGATCAGTAACACCCAGGGAATTGTA-3′). The amplicon and vector (pCS2+ or the myc-tagged version, pCS2+MT) were digested with the same two enzymes and ligated. In addition to serving as templates for *in vitro* transcription of *foxD5aORF* mRNA, they served as templates for PCR-based assembly of chimeric and deletion constructs (Fig. 1A).

To generate a chimeric Engrailed repressor domain/WHD protein (*EnRfoxD5a*), the *foxD5a* WHD was amplified using primers with added restriction sites (*Bsa*BI/XfoxD5a595F: 5′-GTACGTGATGCCAATCTGGTAAAGCCTCCTTAC-3′; *Xho*I/XfoxD5a898R: 5′-AGTTGGCTCGAGCCTCTTAAACCTTTTCCTCCT-3′). The *siamois* DNA-binding domain of the pSia-EnR-XT7 fusion construct (Fan and Sokol, 1997) was excised by digesting with *Bsa*BI and *Xho*I, and the *foxD5a* WHD amplicon (LVK . . . FKR, aa 95–195) was digested and ligated into its place, in-frame and downstream of the Engrailed repressor domain (aa 1–289). To create an N-terminal deletion con-

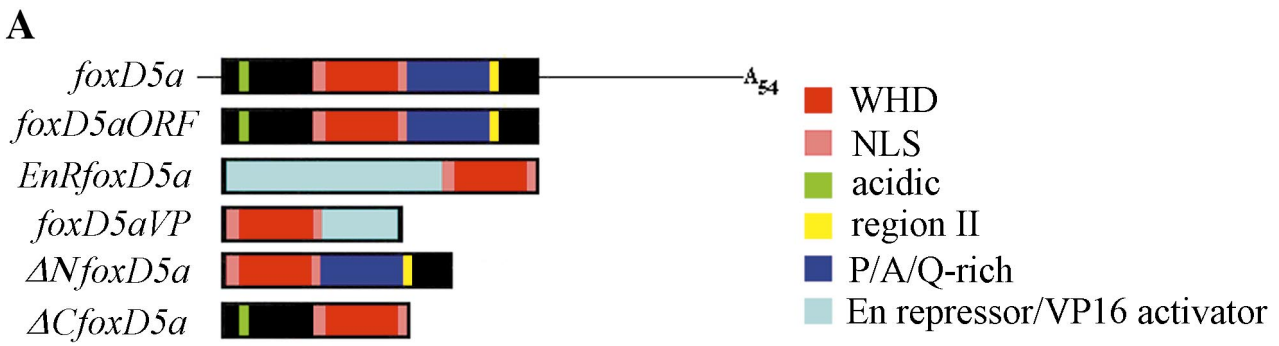


FIG. 1. Sequence analysis of *Xenopus foxD5a*. (A) Schematic representations of *foxD5a* mRNA and expression constructs derived from it. For some experiments, six copies of a myc epitope (not shown) were added to the N-terminus. (B) Alignment of *Xenopus* FoxD5a to some high-scoring BLAST hits. Vertebrate representatives of all Class D subtypes are shown; rat HNF3β, a low-scoring match from Class A, is included to illustrate typical similarities and differences between known gene-repressing WHD proteins (−) and transactivating WHD proteins (+). Identity to FoxD5a is indicated by a dash. WHD boundaries and nuclear localization signals (= = =) are as in Kaufman and Knöchel (1996). Region II boundaries are reduced compared to those reported for HNF3 proteins by Pani *et al.* (1992), reflecting more limited conservation. Two regions of conserved amino acid composition (rather than sequence) are highlighted: acidic residues are shaded in the boxed N-terminal "blob," whereas proline/alanine/glutamine (P/A/Q) residues are shaded in the C-terminal region between the WHD and region II.

struct (Δ*NfoxD5a*), a primer was designed to add a start codon to an amplicon of the WHD/C-terminal region of the ORF (M/LVK . . . GCY, aa 95–352) (ΔNATGXfoxD5a367F: 5′-CCCATCGATTTAAAGCTATGCTGGTAAAGC-3′). *foxD5a* was amplified using this primer in conjunction with a T3 primer. The amplicon and the receiving vector (pCS2+ or pCS2+MT) then were digested with *Cla*I and *Not*I, and ligated. To generate a chimeric protein containing the WHD and VP16 activation domain (*foxD5aVP*), a 3′ *Bst*BI site was added by PCR to the ATG-WHD (M/LVKP. . .) in the N-terminal deletion construct. An ATG-WHD amplicon (MLVK. . .RFKR) and VP16-containing vector (pCS2VP16, coding for PTDV. . .EYGG of VP16 protein) were digested with *Cla*I and *Bst*BI and the WHD restriction fragment was ligated upstream of and in-frame with the VP16 domain. The C-terminal deletion construct (Δ*CfoxD5a*) was generated by amplifying pCS2+MT/*foxD5a* from the SP6 site of the vector through the end of the *foxD5a* WHD (aa 1–195), incorporating an *Xho*I site (*Xho*I/XfoxD5a669R: 5′-AGTTGGCTCGAGCCTCTAAACCTTTTCCT-3′). The amplicon and pCS2+MT were digested with *Bam*HI and *Xho*I, and ligated.

RNA Synthesis and Microinjection

Capped, polyadenylated mRNA was transcribed *in vitro* (mMessage mMachine; Ambion, Austin, TX). Microinjections (1 nl) of *foxD5aORF* (30–500 pg/blastomere), *foxD5aEnR* (100–200 pg), *foxD5aVP* (100–200 pg), Δ*NfoxD5a* (100–200 pg), Δ*CfoxD5a* (100–200 pg), *siamois* (50 pg), *noggin* (5–20 pg), *chordin* (80–120 pg), *cerberus* (75 pg), or *Wnt8* (300–600 pg) were made into blastomeres of stereotypically cleaving embryos obtained from naturally fertilized wild type adults (Moody, 1999). In some cases 100–200 pg green fluorescent protein (GFP) or β-galactosidase (βGal) mRNAs were coinjected as lineage tracers. For each of the *foxD5a* constructs, the myc-tagged version of mRNA also was injected and protein identified by immunohistochemistry (see below) to ensure that each could access the nucleus.

Animal Cap Assays

The animal poles of two- to four-cell stage embryos were microinjected with the above-noted mRNAs. Embryos were cultured to stages 8–8.5, at which time small animal cap explants were removed and cultured in NAM (Messenger and Warner, 1979) to appropriate stages. They were processed for RT-PCR, *in situ* hybridization, or immunohistochemistry with stage-matched uninjected caps and whole embryos for each experiment. All animal cap explant experiments were repeated a minimum of three times. For *in situ* hybridization experiments, caps were sectioned and examined microscopically to ensure accuracy of scoring for positive gene expression.

RT-PCR

RNA was extracted using Purescript (Gentra Systems, Minneapolis, MN) or High Pure (Boehringer Mannheim, Indianapolis, IN) kit protocols; 0.5–1 μg was DNase treated and reverse transcribed using Superscript II RT (LTI). One-tenth (2 μl) of the cDNA reaction was used in the PCR: 94° 1 min/94° 30 s; 55° 30 s; 72° 1 min/72° 2 min. Cycle number was adjusted for each primer set to remain within the linear range of amplification.

In Situ Hybridization

Full-length RNA probes for *foxD5aORF* (sense and antisense), *Xbra* (Smith *et al.*, 1991), *chordin* (Sasai *et al.*, 1994), *engrailed2* (Hemmati-Brivanlou *et al.*, 1991), *Krox20* (Bradley *et al.*, 1992), *Xngnr1* (Ma *et al.*, 1996), *n-tubulin* (Richter *et al.*, 1988), *otx2* (Blitz and Cho, 1995), *sox2* (Penzel *et al.*, 1997), and *sox3* (Penzel *et al.*, 1997; Zygar *et al.*, 1998) were labeled with digoxigenin-UTP or fluorescein-UTP (Boehringer Mannheim). Embryos were fixed in MEMPFA and *in situ* hybridization was performed according to standard protocols (Sive *et al.*, 2000). In some cases βGal mRNA was first detected by standard histochemical protocols. Some

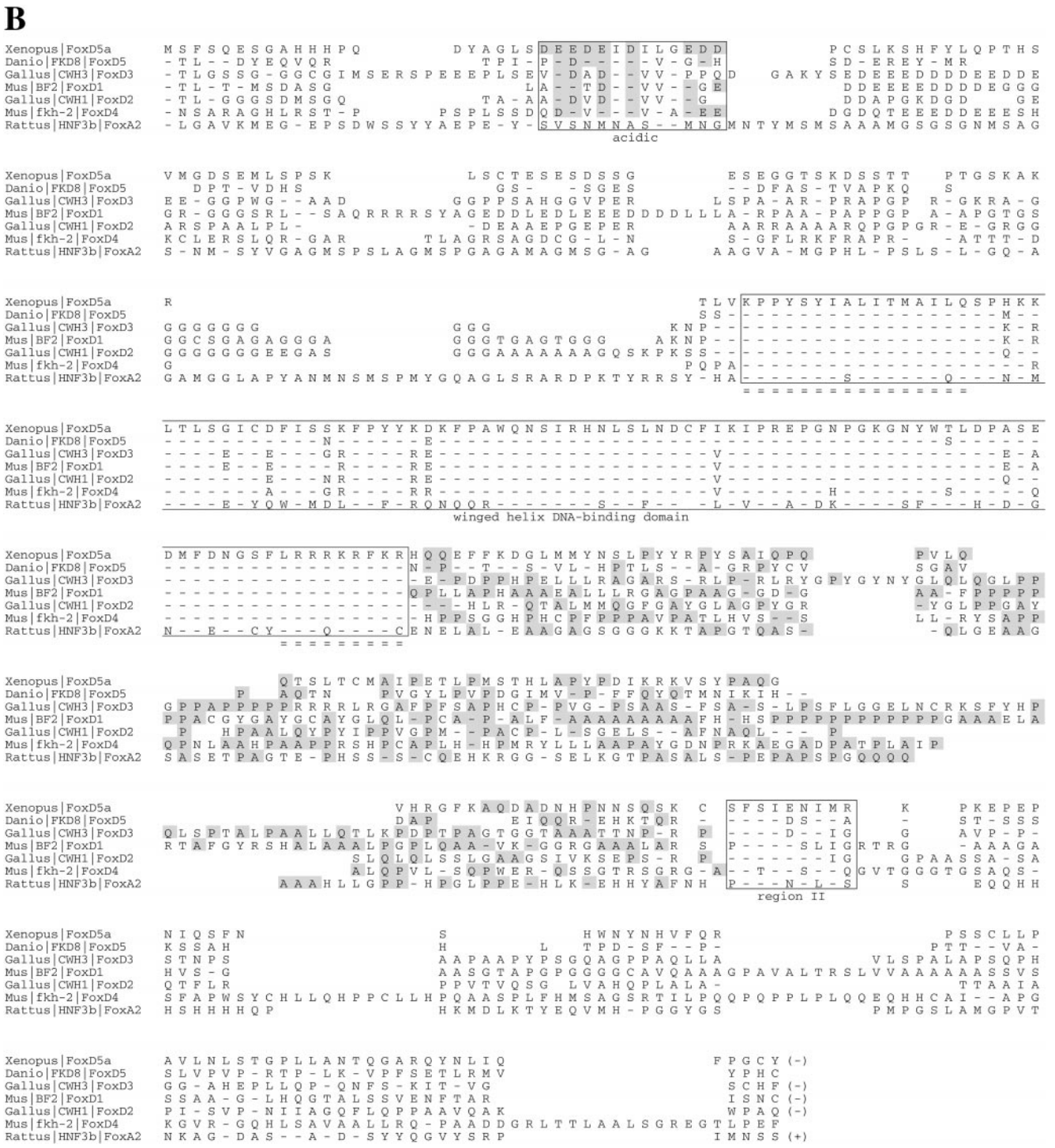


FIG. 1—*Continued*

embryos were subsequently sectioned on a cryostat for refined tissue localization analyses. All animal cap explants were sectioned after *in situ* hybridization to unequivocally confirm gene expression.

Immunohistochemistry

Embryos and explants were fixed in 4% paraformaldehyde, 5% sucrose, 0.1 M PBS, equilibrated in 15% sucrose–PBS, embedded in

OCT medium (Fisher Scientific, Pittsburgh, PA), cryosectioned at 14 μm, and collected on gelatin-coated slides. Sections were subjected to immunohistochemical staining by standard peroxidase–antiperoxidase (PAP) protocols (Moody *et al.*, 1996) or indirect immunofluorescence protocols (Moody *et al.*, 1989). Primary antibodies used were against: myc (Sigma, St. Louis, MO), neural-specific HNK-1 glycoprotein (ATCC, Rockville, MD) (Nordlander, 1993), 12/101 somitic muscle antigen (Developmental Hybridoma Bank, Iowa City, IA) (Kintner and Brockes, 1984), epidermal-specific cytokeratin (Jamrich *et al.*, 1987), and PCNA (Sigma) (Hyde-Dunn and Jones, 1997). In some specimens, sections were incubated in Hoechst reagent (100 μg/ml; Sigma) to visualize cell nuclei.

DNA Synthesis Blockade

To determine whether *foxD5a*-induced expansion of the neural plate is mediated by increased cell division, mitosis was blocked by incubation of the embryos in a cocktail of DNA replication inhibitors (Harris and Hartenstein, 1991). At stages 8, 9, and 10− embryos were cultured in Steinberg's solution containing 20 mM hydroxyurea/150 μM aphidicolin (HUA). Hydroxyurea acts within 2 h, whereas aphidicolin requires 4–6 h for maximal effect. The combination of the two drugs was previously shown to completely block DNA synthesis in *Xenopus* embryos within 3 h of incubation (Harris and Hartenstein, 1991). Embryos were maintained in this cocktail until reaching stages 14/15, at which time they were fixed and processed for *in situ* hybridization.

RESULTS

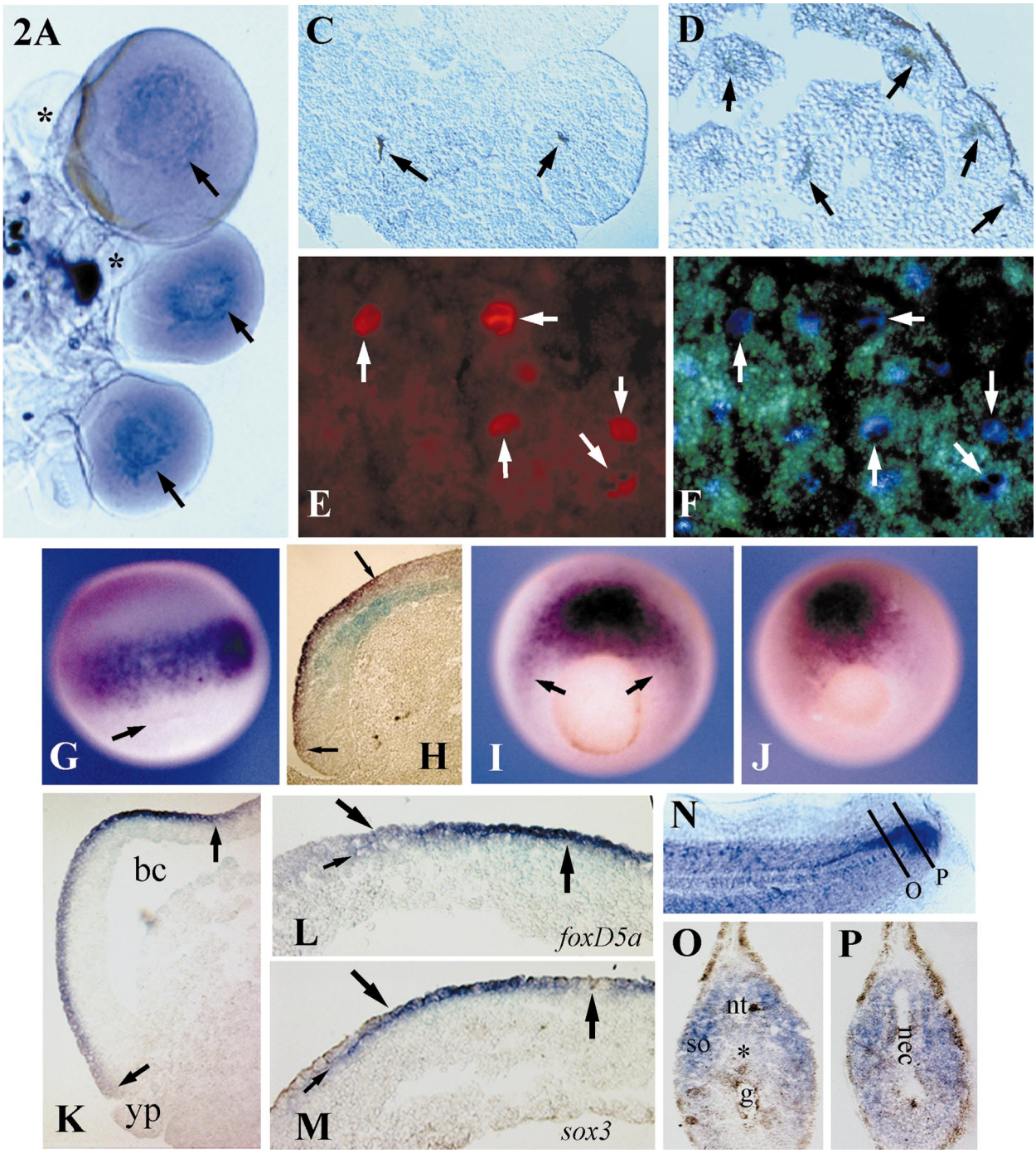
Cloning and Sequence Analysis of Xenopus foxD5a

We obtained an approximately 1.8-kb cDNA (Figs. 1A and 1B) that included sequence identical to a 95-bp PCR fragment deposited previously in GenBank under the locus name XFLIP (King and Moore, 1994). Antisense RNA probes made from our cDNA hybridize to a single ~1.8-kb band in Northern analysis of embryonic RNA (not shown). In accordance with recently proposed nomenclatural conventions for Fork head/Winged Helix proteins this gene is renamed *Xenopus foxD5a*. The longest open reading frame (encoding 354 aa) lies downstream of an 84-nt 5′ untranslated region, and includes a typical winged helix DNA-binding domain. The ORF is followed by a 643-nt 3′ untranslated region that includes a poly(A) signal and A_{54} tail. The 5′ UTR lacks an obvious translation initiation sequence (Kozak, 1990), but of several possible in-frame initiator codons, our choice is supported both by amino-terminal sequence conservation (Fig. 1B) and by software designed to rank potential translational start sites (see Materials and Methods).

Querying GenBank for sequences similar to *foxD5a* preferentially retrieves members of the Fox Class D (formerly Class 5; Fig. 1B) (Kaufmann and Knöchel, 1996), including zebrafish FKD-8, -6, and -9 (Odenthal and Nüsslein-Volhard, 1998), murine and avian Genesis/CWH-3 (Sutton *et al.*, 1996; Freyaldenhoven *et al.*, 1997a), murine and amphibian brain factor (BF)-2 (Hatini *et al.*, 1994; Mariani and Harland, 1998), *Xenopus* XFD-6 (Scheucher *et al.*, 1995), and *Drosophila* FD3 (Häcker *et al.*, 1992). FoxD5a is identical to XFD-12, and highly homologous to its alleles (XFD-12′, 88% amino acid identity; XFD-12″, 71% amino acid identity) (Sölter *et al.*, 1999). Within some classes, subclasses have been distinguished based on characteristic WHD sequence variations (Kaufmann and Knöchel, 1996). Both levels of classification also are generally supported by conservation patterns of regions outside the WHD, which are evident when a comprehensive set of full-length Fox protein sequences is aligned (S. Sullivan, unpublished observation). Such analysis indicates that *Xenopus* FoxD5a (and its alleles XFD-12′ and XFD-12″) and zebrafish FKD-8 belong to a separate, novel subclass, D5 (Fig. 1B).

FoxD5a has several conserved regions outside the WHD (Fig. 1B). The N-terminal "blob" of acidic residues found in FoxD5a also occurs in transcription-repressing WHD proteins such as CWH/Genesis, BF-2 (Class D), and BF-1/qin (Class G), but is absent in HNF3β (Class A), a transcription-activating WHD protein. However, in all WHD proteins characterized so far the major transcription-regulating do-

FIG. 2. Expression of *foxD5a* is both maternal and zygotic. (A) *In situ* hybridization of a piece of ovary using full-length antisense *foxD5a* probe demonstrates that this gene is transcribed in early oogenesis, starting around stage II. Signal is found throughout the cytoplasm and in perinuclear clumps (arrows). Asterisks denote unstained stage I oocytes. (B) Animal (An) and vegetal (Veg) hemispheres of 16-cell embryos were dissected, their RNA extracted and analyzed by PCR. *foxD5a* mRNA, like that of *Xwnt8b* (Cui *et al.*, 1995), is present in the animal hemisphere but not the vegetal hemisphere. *Vg1* is a vegetally localized control (Kessler, 1999). The histone H4 was used as a loading control. (C) Exogenous FoxD5a-myc protein, whose mRNA was injected into the one-cell embryo, can be detected in the nucleus (arrows) as early as the 64-cell stage. Protein was detected by PAP immunohistochemistry. (D) Stage 8 embryo, prepared as in C, showing that exogenous FoxD5a-myc protein appears nuclear (arrows). (E) FoxD5a-myc protein (arrows), detected by a Texas Red secondary antibody, in some cells of a stage 7.5 embryo. (F) Same microscopic field as in E. Hoescht DNA staining (arrows) colocalizes with myc-staining, demonstrating that FoxD5a-myc protein is nuclear. (G) Dorsal view at stage 10. Zygotic *foxD5a* is expressed in a broad band on the dorsal side at a distance from the site of involution at the blastopore (arrow). The unlabeled tissue between the *foxD5a* band and the blastopore is the suprablastoporal endoderm and noninvoluted deep mesoderm (Keller, 1991). (H). Midsagittal section at stage 11 demonstrates *foxD5a* expression in only the superficial cells of the dorsal lip (dark purple between arrows), whereas *chordin* (light blue) is expressed in the underlying mesoderm. (I) Vegetal view at stage 11; *foxD5a* is expressed in the presumptive neural ectoderm of the dorsal lip and weakly in paraxial mesoderm (arrows). (J) Vegetal view at stage 12; *foxD5a* is expressed in the elongating neural ectoderm. (K) Midsagittal section



of stage 13 embryo. *foxD5a* expression extends throughout the anterior–posterior extent of the neural plate (between arrows). bc, blastocoele; yp, yolk plug. (L) Transverse section through a stage 13 embryo. *foxD5a* expression extends from the midline (vertical arrow) nearly to the lateral boundary of the neural plate (oblique arrow). Weak staining in the paraxial mesoderm can be discerned (small horizontal arrow). (M) Transverse section through a stage 13 embryo at level comparable to that in L. *Sox3* expression is absent at the midline (vertical arrow) and the lateral limit of its expression (oblique arrow) marks the lateral boundary of the neural plate. Small horizontal arrow denotes lateral mesodermal expression. (N) Stage 33/34 embryo demonstrating *foxD5a* expression in the tail bud. Lines denote level of sections shown in O and P. (O) Transverse section through rostral tail bud demonstrating *foxD5a* expression throughout the newly formed neural tube (nt) and the paraxial mesoderm (so). Note lack of staining in the notochord (asterisk) and postanal gut tube (g). (P). Transverse section caudal tail bud demonstrating *foxD5a* expression throughout the tissue surrounding the neurenteric canal (nec), which comprises both of presumptive neural and mesodermal components.

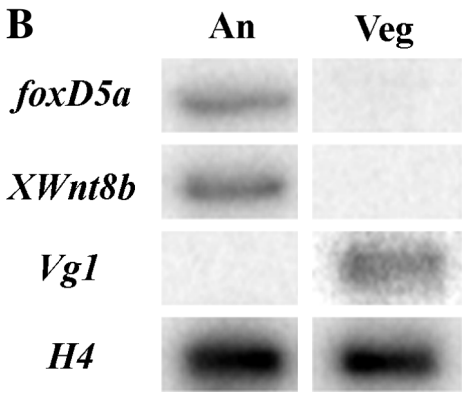


FIG. 2—*Continued*

mains reside in the C-terminal third of the protein (e.g., Pani *et al.*, 1992; Li *et al.*, 1995; Schuddenkopf *et al.*, 1996; Mariani and Harland, 1998; this study). Two features of the C-terminal third of FoxD5a [the so-called "region II" (Lai *et al.*, 1991) and a proline/alanine/glutamine (P/A/Q)-rich region flanked by region II and the WHD] have been noted in both repressing and activating WHD proteins (Fig. 1B), but their importance varies in the two functional classes. In HNF3β normal levels of transcriptional activation require the presence of region II plus two other activating regions outside the WHD that are not conserved in repressing WHD proteins. Deleting the P/A/Q region of HNF3β does not affect its ability to activate transcription (Pani *et al.*, 1992). By contrast, the P/A/Q-rich region of CWH3 is necessary and sufficient for full transcriptional repression. In constructs lacking this region, transcription-repressing activity is reduced approximately 30-fold, but does not diminish further when region II also is deleted (Freyaldenhoven *et al.*, 1997b). P/A/Q enrichment also is characteristic of the repressor domains of the *Drosophila* transcription factors even-skipped, Kruppel, and Engrailed (reviewed in Hanna-Rose and Hansen, 1996). Thus, FoxD5a's homology to repressing Class D WHD proteins and the presence of a P/A/Q-rich region in its C-terminal third indicate that it could act as a repressor of transcription.

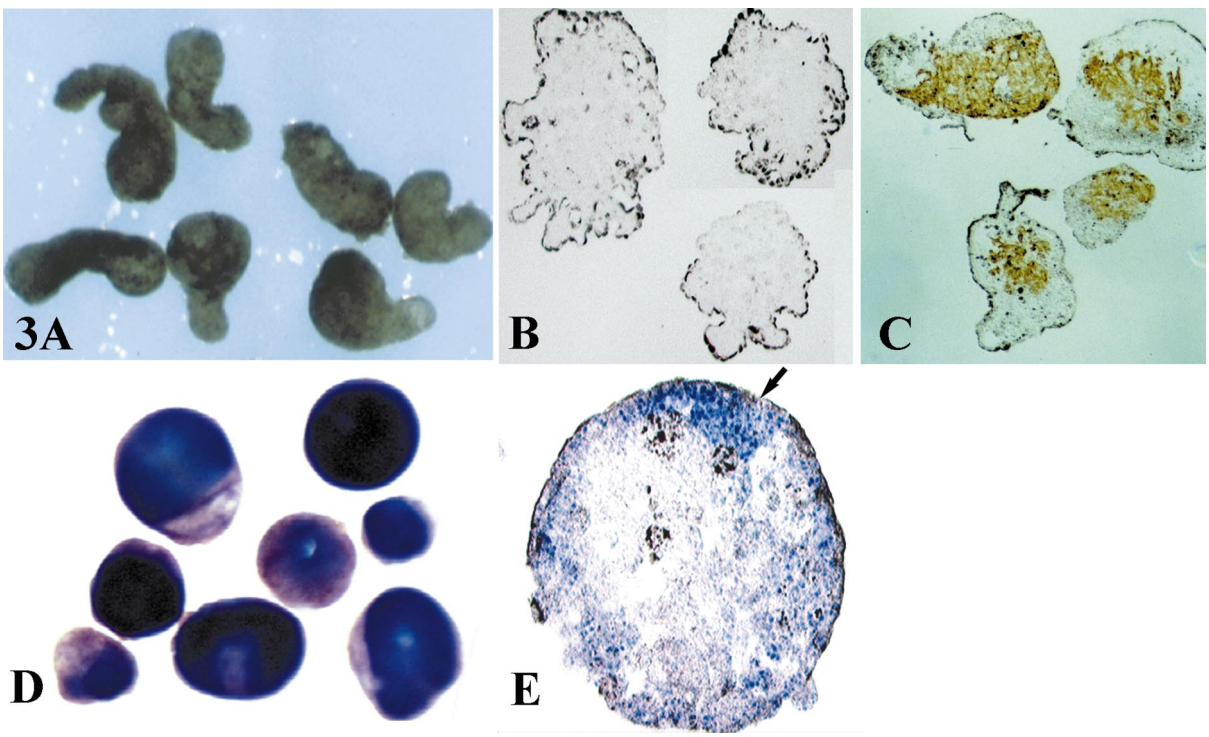


FIG. 3. Expression of *foxD5a* in animal cap explants causes dorsal axis differentiation. (A) Animal cap explants injected with *foxD5a* mRNA at the two-cell stage elongate after 2 days of culture. (B) Tissue sections of control, uninjected explants cultured for 2 days and stained for the 12/101 antigen. They do not express this somitic muscle marker. (C) Tissue sections of elongated *foxD5a*-injected explants cultured for 2 days express the 12/101 antigen (brown PAP reaction product). (D) *foxD5a*-injected explants cultured for 1 day have not yet elongated, but express the early neural plate marker *sox3* (blue). (E) Tissue section of a *foxD5a*-injected explant cultured for 1 day expressing *sox2* (blue). Arrow indicates a cluster of sox-2-positive cells. (F) *foxD5a*-injected animal cap explants express some mesoderm markers, one neural inducer, and some neural markers. Explants analyzed for Xbra1, Xnr3, noggin, and geminin were collected at stage 12; those for Xngnr1 were collected at stage 14; those for m-actin and n-tubulin at stages 18–20. Un, uninjected, stage-matched control explants; WE, stage-matched whole embryo with (+) or without (−) reverse transcription. EF1a served as a positive control.

TABLE 1
Frequency of Dorsal Axial Phenotypes Resulting from Expression of *foxD5a* Constructs

	Control % (n)	*foxD5a* % (n)	*EnRfoxD5a* % (n)	*FoxD5aVP* % (n)	Δ*NfoxD5a* % (n)	Δ*CfoxD5a* % (n)
Animal cap elongation	4.6 (263)	77.2 (158)	68.2 (85)	0.0 (140)	69.2 (107)	0.9 (109)
Animal cap, 12/101	0.0 (21)	92.0 (27)	83.7 (43)	0.0 (14)	76.7 (30)	0.0 (51)
Animal cap, *sox3*	28.6 (35)	78.9 (52)	ND	ND	86.3 (51)	ND
Animal cap, *sox2*	0.0 (47)	73.0 (63)	15.6*(32)	0.0 (35)	27.3*(11)	0.0 (33)
Ectopic ventral *sox3*	0.0 (84)	0.0 (69)	4.0 (124)	0.0 (25)	0.0 (79)	0.0 (47)
Expanded neural plate (*sox3*)	6.7 (75)	60.8 (120)	60.9 (90)	0.0 (45)	51.4 (35)	6.3 (48)
Expanded otx2	0.0 (53)	61.4 (114)	60.7 (23)	ND	66.7 (24)	8.8 (45)
Expanded chordin	0.0 (22)	0.0 (70)	0.0 (21)	0.0 (21)	ND	ND
Repressed en2	0.0 (67)	67.3 (52)	54.4 (66)	0.0 (10)	46.2 (39)	8.3 (36)
Repressed Krox20	0.0 (12)	82.5 (57)	62.5 (40)	0.0 (9)	62.5 (32)	5.0 (40)
Repressed Xngnr1	0.0 (61)	89.6 (77)	65.5 (29)	ND	46.2 (13)	6.3 (16)
Repressed NeuroD	0.0 (29)	83.3 (6)	50.0 (16)	ND	ND	ND
Repressed *n*-tubulin	0.0 (33)	52.5 (61)	57.7 (26)	ND	ND	ND

Note. ND, experiment not done.
* Although these values are low in comparison to those for the *foxD5a* construct, they are significantly different from control value ($P < 0.01$) using χ^2 analyses.

Expression Is Both Maternal and Zygotic

foxD5a mRNA is detected in the oocyte as early as stage II (Fig. 2A). It is present throughout the cytoplasm and in perinuclear clumps. Maternal transcripts continue to be detectable after fertilization, and are localized to the animal hemisphere, as demonstrated by colocalization with *Xwnt8b* (Cui *et al.*, 1995) but not *Vg1* mRNA (Fig. 2B). The animal hemisphere levels are low in comparison to zygotic expression, but are comparable to that of *Xwnt8b*, which is postulated to function in dorsal axis formation (Cui *et al.*, 1996). Some maternal proteins function by accessing the nucleus prior to the onset of zygotic transcription at MBT (Schneider *et al.*, 1996; Larabell *et al.*, 1997). Consistent with this possibility, a myc-tagged *foxD5a* construct injected at the one-cell stage produces protein that begins to accumulate in the nucleus as early as the 64-cell stage (Fig. 2C), and is nearly all nuclear by stages 7.5–8 (Figs. 2D–2F). These data do not prove a maternal function for *foxD5a*, but clearly demonstrate that its mRNA can be translated and efficiently translocated to the nucleus prior to zygotic gene activation.

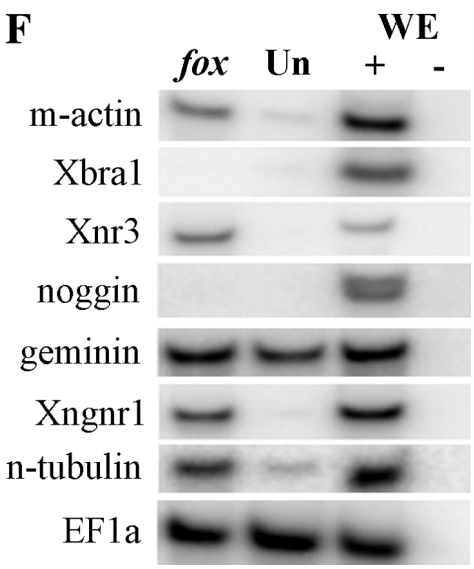


FIG. 3—*Continued*

The zygotic expression of *foxD5a* is very similar, but not identical, to that reported for its *XFD-12′* allele (Solter *et al.*, 1999; Fetka *et al.*, 2000). Here we extend those observations and note some of the differences. Zygotic transcripts are first detected between stage 8 and stage 9 (pregastrula) in a broad equatorial band on one side of the embryo. At the onset of involution (stage 10−) this band can be identified as the presumptive neural ectoderm (Fig. 2G); *foxD5a*-expressing cells do not populate the involuting marginal zone at the dorsal lip, which consists of endoderm and deep mesoderm (Keller, 1991). By stage 10.5, *foxD5a*-expressing cells reach the blastopore, but do not involute; *foxD5a* expression is confined to the superficial layer overlying the chordin-expressing midline mesoderm (Fig. 2H). By stage 11, however, a low level of expression is detected in the paraxial mesoderm (Figs. 2I and 2L), but unlike the *XFD-12′* allele no expression was observed in the notochord. As the neural ectoderm converges and extends to elongate, the domain of *foxD5a*-expressing cells similarly lengthens (Fig. 2J), such that by stage 13 they occupy the entire rostral–caudal extent of the neural plate (Fig. 2K). At

this time *foxD5a* expression also encompasses 10–16 cell diameters across the medial–lateral expanse of the neural plate (Fig. 2L), nearly matching the extent of *sox3* expression at this same stage (Fig. 2M). Thus, *foxD5a* is expressed by nearly all neural plate cells, not just the midline cells as reported for the *XFD-12′* allele. As the neural folds begin to elevate, *foxD5a* expression is lost first at the spinomedullary junction, progressively decreasing in both rostral and caudal directions. Concomitantly it is lost laterally and is maintained the longest in cells near the neural plate midline. Expression is extinguished as the neural tube closes; we did not observe expression at the midbrain–hindbrain junction as described for the *XFD-12′* allele.

After stage 20, *foxD5a* expression is reinitiated at a second site, the growing tail bud, whose tissues are undergoing secondary gastrulation (Fig. 2N). *foxD5a* is expressed in the entire neural tube just rostral to the neurenteric canal (Fig. 2O), not just the roof plate as described for *XFD-12′*. It also is expressed strongly in the paraxial mesoderm, but not the notochord (Fig. 2O). At the level of the neurenteric canal, it is expressed throughout the presumptive neural–mesodermal tissues (the NM region of Beck and Slack, 1998) (Fig. 2P). These minor differences in expression domains may result from the fact that the *XFD-12′* allele is only 81% identical at the nucleotide level to *foxD5a*. Subtle differences of allelic expression patterns were previously reported for a few other genes in *Xenopus* (e.g., *XFKH4* versus *XBF1*) (Dirksen and Jamrich, 1995; Bourguigon *et al.*, 1998); however, it is not clear how these differences might affect embryonic patterning.

foxD5a Upregulates Mesodermal, Neural-Inducing, and Early Neural Plate Genes

The restriction of maternal mRNA to the animal hemisphere and zygotic mRNA to the dorsal blastopore lip and tail bud suggests a role for *foxD5a* in dorsal axis formation. To determine potential targets in this pathway, *foxD5aORF* mRNA (hereafter called *foxD5a* RNA) was expressed in animal cap explants. A significant proportion of injected caps cultured for 2 days elongated (Table 1; Fig. 3A), a phenotype indicative of dorsal axial tissue differentiation (Symes and Smith, 1987; Wilson and Keller, 1991). *foxD5a*-injected caps expressed both mesodermal and neural markers. By *in situ* analyses we detected induction of the 12/101 muscle-specific antigen in 2-day explants, and *sox2* and *sox3* in 1-day explants (Table 1; Figs. 3C–3E). There is a low level of maternal *sox3* expression (Penzel *et al.*, 1997) in controls, but the frequency of its detection in explants increases significantly after *foxD5a* mRNA injection (Table 1; $P < 0.05$, χ^2 analysis). By RT-PCR analyses we detected upregulation of some mesodermal genes (m-actin, Fig. 3F), whereas others are not consistently changed from control cap levels (*Xbra1*, Fig. 3F; also *eFGF* and *Xbra3*, data not shown). One neural-inducing gene, *Xnr3*, which is activated by Wnt-signaling during gastrulation (McKendry *et al.*, 1997), is upregulated by *foxD5a*, whereas *noggin* expression is not (Fig. 3F). *Geminin*, one of the earliest expressed neuralizing molecules (Kroll *et al.*, 1998), has a common expression pattern with *foxD5a* but is not upregulated above the maternal levels present in control caps. However, two other neural-specifying genes, *Xngnr1* and *n-tubulin*, are consistently induced (Fig. 3F). These results, as well as the formation of a partial secondary axis after ventral injection (see below), suggest that *foxD5a* may have a role in dorsal axis formation by influencing the expression of a limited set of mesodermal, neural-inducing, and neural-specifying genes.

Regulation of Zygotic foxD5a Expression

The zygotic expression of *foxD5a* in the neural ectoderm of the dorsal lip may result from Wnt-initiated signaling from the Nieuwkoop Center, a dorsal vegetal region that establishes the Organizer (Nieuwkoop, 1973). Consistent with this possibility, UV-irradiation of the fertilized egg that prevents the formation of the Nieuwkoop Center substantially reduces or extinguishes (92%; n = 50) zygotic *foxD5a* expression, and cleavage stage exposure to LiCl that dorsalizes embryos by activating the Wnt pathway (Kao and Elinson, 1988; Klein and Melton, 1996) expands zygotic *foxD5a* expression (94%, n = 50). Injection of Wnt8 mRNA at levels that induce secondary axes in whole embryos (92.5%, n = 53) also induces *foxD5a* expression in ectopic ventral fields (Fig. 4A; 92.3%, n = 26). However, this level of Wnt8 mRNA, which is effective as a direct neural inducer (Baker *et al.*, 1999), only weakly induces *foxD5a* expression in animal cap explants (Fig. 4D). In contrast, ectopic expression of *siamois*, which is regulated by the Wnt pathway and expressed in the Nieuwkoop Center (Lemaire *et al.*, 1995; Brannon and Kimelman, 1996; Carnac *et al.*, 1996; Kessler, 1997; Fan *et al.*, 1998), strongly activates *foxD5a* transcription. Injection of *siamois* mRNA at levels that induce secondary axes in whole embryos (77.4%, n = 62) also induces *foxD5a* expression in ectopic ventral fields (Fig. 4B; 67%, n = 18) and causes robust induction in animal cap explants (Fig. 4D).

Analysis of sectioned explants subjected to *in situ* hybridization concurs with these results: Wnt-injected caps are positive for *foxD5a* mRNA only infrequently (15.6%, n = 32), whereas *siamois*-injected caps consistently express *foxD5a* mRNA (81.8%, n = 44). Siamois induces Organizer genes, including several neural inducers (Carnac *et al.*, 1996). To test whether neural-inducing, anti-BMP molecules activate the expression of *foxD5*, animal caps were injected with levels of mRNAs that induce secondary axes in whole embryos (noggin, 89%, n = 46; cerberus, 84%, n = 38; chordin, 75%, n = 28). RT-PCR analyses demonstrate that under conditions in which a neural marker (*Xngnr1*) is induced, *foxD5a* is moderately induced by cerberus and noggin but not by chordin (Fig. 4D). Analyses of similarly injected, sectioned explants subjected to *in situ* hybridization also showed moderate induction by cerberus (34.5%, n = 29), but no induction by noggin (0%, n = 79) or chordin

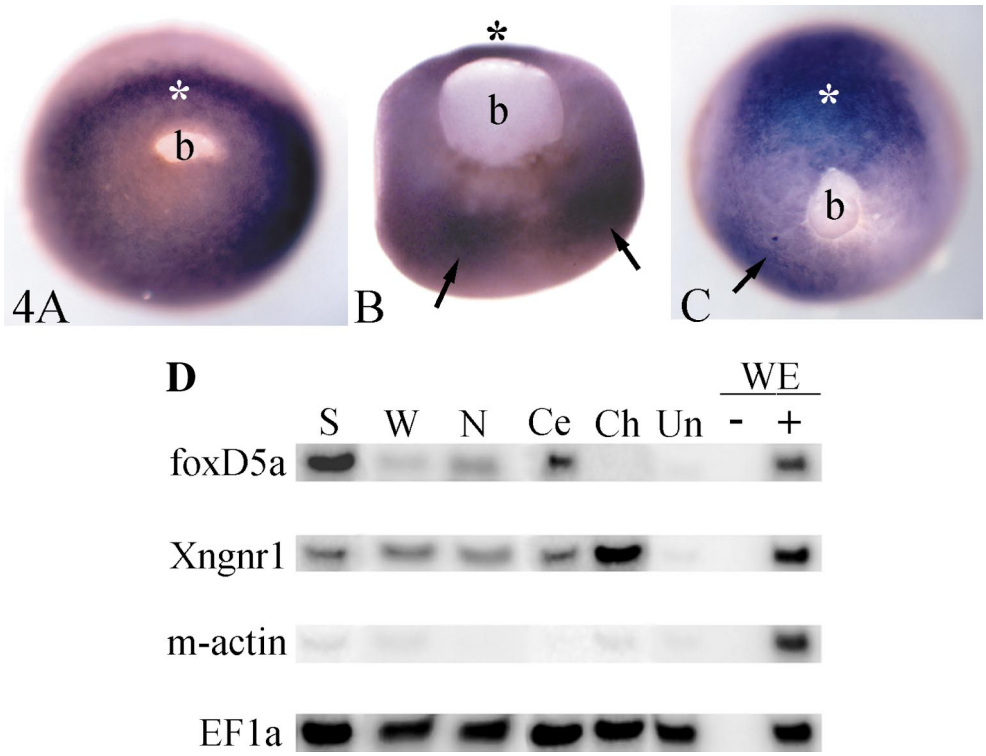


FIG. 4. *siamois* and select neural inducers upregulate *foxD5a* zygotic expression. (A) Ectopic circumblastoporal expression of *foxD5a* (purple) after bilateral injection of *Wnt8* mRNA. Asterisk denotes the normal dorsal expression domain of *foxD5a.* Compare to uninjected controls (Figs. 2I and 2J). Vegetal view; b, blastoporal region. (B) Ectopic ventral expression of *foxD5a* (arrows) after bilateral injection of *siamois* mRNA. Asterisk denotes the normal dorsal expression of *foxD5a* in the dorsal lip at stage 12. Ventral, vegetal view. (C) Ectopic ventral expression of *foxD5a* (arrow) after bilateral injection of *noggin* mRNA. Asterisk denotes the normal dorsal expression domain of *foxD5a.* Ventral view. (D) *foxD5a* expression in animal caps is upregulated robustly by siamois (S) and cerberus (Ce), moderately by Wnt8 (W) and Noggin (N), and not by chordin (Ch), under conditions that induce neural genes (*Xngnr1*) and not muscle genes (*m-actin*). WE, whole embryo with (+) or without (−) reverse transcription. Un, uninjected control caps. EF1a served as a positive control. All explants and whole embryos were harvested between stages 12/13, when *foxD5a* expression in the whole embryo is maximum.

(0%, n = 46). However, like *siamois* and *Wnt8, noggin* induced ectopic ventral expression of *foxD5a* in whole embryos (Fig. 4C). These data demonstrate that those genes shown to be downstream of axis-inducing Wnt signaling (*siamois, cerberus*) regulate *foxD5a* expression, whereas neural-inducing BMP antagonists are variably less effective. Consistent with this trend, the weak induction of *foxD5a* in Wnt8-injected animal caps may reflect its activity under these conditions as a direct neural inducer (Baker *et al.*, 1999), rather than as an activator of *siamois.*

foxD5a Expression Expands Dorsal Axial Tissues

To elucidate the role of *foxD5a* in axial tissue formation, mRNA was ectopically expressed in whole embryos. A dose–response relationship was detected. Injection of <30 pg in a single 16-cell blastomere showed no phenotype, 50 pg/cell showed modest effects, 100–200 pg/cell showed consistent effects in >60% of embryos, 300 pg/cell showed strong effects in all embryos with some indication of cell damage (see Moody, 1999 for discussion of these signs), and >300 pg/cell showed consistent loss of injected cells from the embryo, indicating toxicity. Thus, for all results described below, 100–200 pg per 8- to 16-cell blastomere was injected.

Because *foxD5a* initiates mesodermal and neural gene expression in animal caps, we tested whether an ectopic secondary axis formed after mRNA injection into single ventral blastomeres. *foxD5a*-expressing cells, identified by the coexpression of GFP, were located in the appropriate position for the injected lineage, as determined by comparison to fate maps of normal embryos (Moody, 1987a,b), indicating that cell fate per se was not altered. Secondary axial structures consistently formed from tissue at the injection sites (73.3%, n = 90; Fig. 5A), but these axes were not complete. Neither of two neural-specific markers, HNK1 (not shown) and *sox3* mRNA (Table 1), were detected at the injection sites. Thus, in the ventral domain of the embryo, in contrast to animal caps or dorsal blastomere injections (see below), *foxD5a* alone is not sufficient to

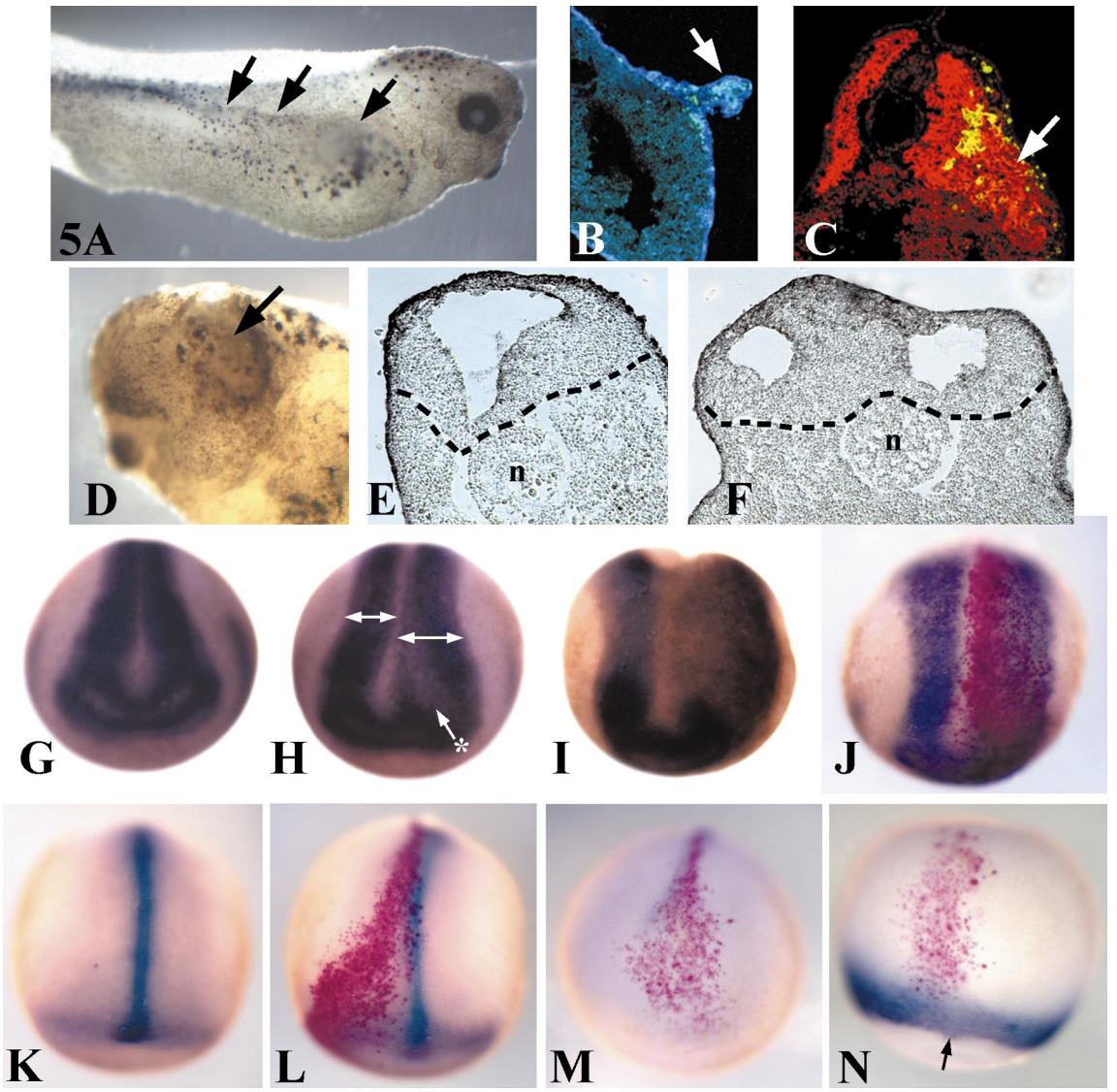


FIG. 5. Ectopic expression of *foxD5a* causes expansion of axial tissues in embryos. (A) Embryo in which *foxD5a* mRNA was injected into one ventral vegetal blastomere. A partial secondary axis is apparent (arrows). (B) Tissue section from a similarly injected embryo demonstrating expansion of the epidermis, marked by keratin immunostaining (dark blue), in the partial axis (arrow). Double-labeled cells expressing *foxD5a*, which were marked by GFP, appear light blue within the ectopic tissue. (C) Tissue section from a similarly injected embryo demonstrating expansion of the somitic mesoderm, marked by 12/101 immunostaining (red), in the partial axis (arrow). Cells expressing *foxD5a*, marked by GFP, are double-labeled (yellow) within the expanded somite. (D) Embryo in which *foxD5a* mRNA was injected into one dorsal midline, animal blastomere. A tissue bulge emanating from the neural tube (arrow) obscures the eye. (E) DIC image of transverse tissue section of hindbrain from a similar embryo. Neural tube is thicker on the injected side (right) compared to that on the control side (left). Dashed line demarcates the basal lamina of the neural tube. n, notochord. (F) DIC image of transverse tissue section of hindbrain from an embryo injected bilaterally with *EnRfoxD5a* mRNA. Neural tube is expanded on both sides to appear duplicated. Dashed line denotes basal lamina of the neural tube. There is only one notochord (n). (G) Anterior view of whole-mount *in situ* hybridization preparation of control embryo (stage 15) stained for *sox3* expression, which is absent at the midline. (H) Anterior view of a *foxD5a* mRNA-injected embryo (stage 15) stained for *sox3* expression. The neural plate on the injected side (right) is about 25% broader than that on the uninjected side (left), as measured from the unstained midline. The clearing of sox3 expression in the anterior neural plate (asterisk) may indicate a secondary floor plate. (I) Dorsal view of a *foxD5a* mRNA-injected embryo (stage 14) stained for *sox3* expression. The neural plate on the injected side (right) is nearly twice as broad as that on the uninjected side (left). (J) Dorsal view of stage 14 *foxD5a*-injected embryo treated with HUA at stage 10−. The neural plate is demarcated by *sox3* expression (blue) and the *foxD5a*-expressing cells are red (*β*gal). The injected side of the neural plate (right) is nearly 50% broader than the control side (left). (K) Dorsal view of control embryo (stage 15) stained for chordin expression (blue). Posterior is to the top. (L) Dorsal view of *foxD5a*-injected embryo (stage 15). Chordin expression (blue) does not expand laterally, concomitant with *foxD5a* expression (red). (M) Anterior view of stage 14 *foxD5a*-injected embryo. *Xbra1* expression (blue) is confined to the periblastoporal region (not seen from this view), and does not expand into the anterior domain of the *foxD5a*-expressing cells (red). (N) Dorsal view of stage 11 *foxD5a*-injected embryo. *Xbra1* expression (blue) encircles the blastopore (arrow) and does not extend anteriorly into the domain of the *foxD5a*-expressing cells (red).

convert ectoderm to neural tissue. However, both ectoderm and mesoderm dramatically expanded in the secondary axial structures. Epidermis (Fig. 5B) and somitic mesoderm (Fig. 5C), identified by tissue-specific markers, were expanded in regions of GFP-labeled *foxD5a*-expressing cells (70.4%; n = 54).

In contrast, injection of *foxD5a* mRNA into single dorsal animal blastomeres, which are the major progenitors of the nervous system (Moody, 1987a,b), caused hypertrophy of the neural ectoderm. The morphology of the anterior CNS was dramatically disrupted (86.3%, n = 51), with flattened forebrains and severely disrupted eyes (Fig. 5D). Histological analysis of these embryos suggests that this phenotype is the result of a disturbance in head morphogenesis, which in turn is the result of significant expansion of the neural ectoderm on the injected side of the embryos (Figs. 5E and 5F). When examined at neural plate stages, there appeared to be a duplication or expansion of the anterior neural plate, which was confirmed by an expansion of *sox3* and *otx2* expression in a significant number of embryos (Table 1; Figs. 5H, 5I, and 6B). The width of the *sox3*-expression domain in the neural plate on the injected (marked by lineage tracer) versus uninjected side was measured using an eyepiece micrometer. In control embryos there was an average difference of 1.3% between the two sides (n = 33), whereas in *foxD5a*-injected embryos the injected side was enlarged by an average of 22.7% (n = 51). It should be noted that in no case was ectopic *sox3* expression observed in those parts of the *foxD5a*-expressing clone that were within the epidermis, confirming the observations from ventral mRNA injections that this gene does not directly convert epidermis to neural ectoderm.

To determine whether the expansion of the neural ectoderm was the direct result of increased proliferation, embryos were incubated in a cocktail of DNA synthesis inhibitors (HUA) at the onset of *foxD5a* zygotic expression. This cocktail blocks DNA synthesis completely in *Xenopus* embryos within 3 h of incubation (Harris and Hartenstein, 1991). In accord with previous reports (Harris and Hartenstein, 1991; Bellefroid *et al.*, 1998; Moody *et al.*, 2000), embryos treated at stage 8 did not gastrulate, and embryos treated at neural plate stages displayed reduced eyes and CNS, indicating that the treatment was effective. Nonetheless, *sox3* expression on the injected side was enlarged (by 23.5%, n = 59) in embryos treated with HUA beginning at either stage 9 or stage 10− (Fig. 5J). Because it is possible that *foxD5a* causes an increase in proliferation during the 3-h interval required for the drug treatment to be effective, an alternate method for detecting whether *foxD5a* increases cell proliferation was employed. Embryos were immunostained for PCNA, an auxiliary protein of DNA polymerase specifically observed in proliferating cell nuclei (Hyde-Dunn and Jones, 1997). The number of cells having nuclear PCNA staining was counted in several microscopic fields that contained *foxD5a*-expressing cells (marked with a lineage tracer). Overall, *foxD5a*-expressing cells accounted for 11.4% of the PCNA-labeled cells (34 nonadjacent tissue sections from eight embryos were analyzed). In one embryo the total number of PCNA-positive cells was counted, of which *foxD5a*-expressing cells accounted for 14.9%. Considering that *foxD5a*-expressing cells represent 12.5% of the total cells in the embryo (because an 8-cell blastomere was injected), these data confirm that *foxD5a* does not significantly increase the proportion of cells engaged in proliferation.

Because *foxD5a* can upregulate some mesodermal genes in animal caps and secondary axes (Figs. 3 and 5C), we tested whether the expansion of the neural plate is caused by the expansion of underlying, neural-inducing mesoderm. Neither chordin-expressing midline mesoderm (Fig. 5L; Table 1) nor Xbra1-expressing paraxial mesoderm (0%, n = 43; Figs. 5M and 5N) was expanded. These data indicate that *foxD5a* expands the neural plate downstream of the induction of neural ectoderm by mesoderm.

foxD5a Expression May Expand the Neural Precursor Pool by Inhibiting Differentiation

Initial neural induction appears to first confer an "anterior" identity to the responsive neural ectoderm that is later transformed by posterior signals into the different regions of the CNS (Gould and Grainger, 1997; Nieuwkoop, 1997). We investigated whether the expansion of the neural plate in *foxD5a*-injected embryos is the result of a prolongation of the initial, anterior state. Consistent with this hypothesis, *otx2* expression, which normally demarcates the forebrain region of the neural plate (Fig. 6A), was frequently expanded (Table 1), in a few cases all the way to the closing blastopore (Figs. 6B and 6C). Additionally, the expression domains of *en2* (midbrain/hindbrain boundary marker, Fig. 6D) and *Krox20* (hindbrain marker, Fig. 6F) were more posterior on the injected side of a small number of embryos (13.9%, n = 79; 15.7%, n = 57, respectively). However, the expression of these later-activated genes mostly was repressed in *foxD5a*-expressing cells (Figs. 6D–6G; Table 1). Adjacent cells not expressing exogenous *foxD5a* were unaffected and expressed the appropriate markers. The expansion of an early anterior marker concomitant with the repression of later-expressed posterior markers only in those cells expressing exogenous *foxD5a* suggests that *foxD5a* does not alter the patterning of the neural plate per se, but maintains cells in an immature state. In concordance, the expression of *Xngnr1* (Figs. 6H and 6I) and *NeuroD* (Table 1), proneural bHLH genes that act to define neuronal progenitor cells (Ma *et al.*, 1996; Lee *et al.*, 1995) were similarly repressed. A neural differentiation gene, *n-tubulin* (Richter *et al.*, 1988), was repressed in *foxD5a*-expressing cells at the onset of its expression in the neural plate or trigeminal ganglion (Figs. 6J and 6K; Table 1). However, if embryos were allowed to develop to neural fold and tube stages (17–20), when large numbers of primary neurons are postmitotic and differentiating, ectopic *n-tubulin*-expressing neurons were ob-

served within the domain of *foxD5a*-expressing cells (57.8%, n = 45; Fig. 6L). Taken together, these results suggest that the early repression of proneural and differentiation genes by *foxD5a* expands the neural plate precursor pool and ultimately leads to greater numbers of differentiating neurons.

foxD5a Acts as a Transcriptional Repressor via the C-Terminal Domain

Different Fox proteins can function as either transcriptional activators or transcriptional repressors. Several family members of Class D were previously shown to function as transcriptional repressors (Sutton *et al.*, 1996; Freyaldenhaven *et al.*, 1997b; Mariani and Harland, 1998), and the structure of *foxD5a* suggested it may function as a repressor as well (Fig. 1B). To directly determine this, the *foxD5a* WHD was cloned downstream of the transcription-repressing domain of *Drosophila engrailed* (Fig. 1A). Ectopic expression of *EnRfoxD5a* mRNA mostly mimicked the effects of ectopic expression of *foxD5a* mRNA. Expression of *EnRfoxD5a* mRNA in dorsal blastomere lineages caused morphogenetic disruption in the head (84%; n = 25) and expanded the neural ectoderm, as indicated by *sox3* expression (Table 1) and histological analysis of the neural tube (Fig. 5F). When expressed in the ventral marginal zone, it caused partial secondary axes but did not induce *sox3* expression (Table 1). Animal cap explants injected with *EnRfoxD5a* mRNA elongated and expressed 12/101 and *sox2* (Table 1). Dorsal expression of *EnRfoxD5a* mRNA expanded the neural plate (*sox3* and *otx2*) in the absence of axial mesoderm expansion (chordin), and repressed later neural markers (Table 1). In contrast, the *foxD5aVP* transcriptional activator construct (Fig. 1A) caused none of these changes (Table 1). Because *EnRfoxD5a* effects were similar to those of the *ORF* construct and the *foxD5aVP* construct caused no detectable phenotypes, we conclude that *foxD5a* functions predominantly as a transcriptional repressor.

There are conserved domains in both the N-terminal and C-terminal portions of FoxD5a that in other Fox proteins mediate transcriptional regulation of target genes (Fig. 1). To identify which portion of the FoxD5a protein is responsible for its downstream effects, deletion mutants were constructed (Fig. 1A). Expression of Δ*NfoxD5a* in both animal caps and whole embryos mimicked most of the effects of the *ORF* construct, including animal cap elongation and expression of mesodermal and neural markers, expansion of the neural plate (*sox3* and *otx2*), and repression of later neural markers (Table 1). Conversely, expression of the Δ*CfoxD5a* construct had minimal effects on any of these (Table 1). These results demonstrate that the region primarily responsible for the ectopic expression phenotypes of *foxD5a* lies in the C-terminal domain of the protein.

DISCUSSION

FoxD5a Acts as a Transcriptional Repressor

Fox proteins have been classified by relatively minor differences in the highly conserved DNA-binding domain (Kaufmann and Knöchel, 1996; Kaestner *et al.*, 2000). Whereas variations in the WHD mediate differential binding of Fox proteins to DNA sequences (Kaufmann *et al.*, 1995; Marsden *et al.*, 1997), and thus determine which genes will be regulated, the regulatory function itself resides in the flanking N- and C-terminal domains. In our assays a *foxD5a*-Engrailed repressor chimeric construct, an N-terminal deletion construct, and full-length *foxD5a* all yield qualitatively similar phenotypes, whereas a C-terminal deletion construct and a *foxD5a-VP16* transactivating chimeric construct have virtually no effects. These results indicate that FoxD5a acts as a repressor, and that the major locus of gene-repressing activity is found in the C-terminal third of the protein. Although N-terminal "acid blobs" are characteristic of the repressing Class D and Class G WHD proteins (Fig. 1B and S. Sullivan, personal observation), they are also a feature of many transactivating proteins, where in fact they were first identified (reviewed in Ptashne, 1988). The N-terminal domain may contribute a minor component to the repressive activity in *Xenopus* BF-2 (Mariani and Harland, 1998), but there and elsewhere among WHD proteins (e.g., Freyaldenhoven *et al.*, 1997b) it appears subsidiary to activity imparted by the C-terminal domain.

Although region II is the best-conserved sequence in the C-terminal third of FoxD5a, it is unlikely to be the major effector of gene repression. In deletion assays to date of both activating and repressing WHD proteins, region II has evinced an apparently modulatory or coregulatory role, rather than a function-determining role (Pani *et al.*, 1992; Freyaldenhoven *et al.*, 1997b). Moreover, while it is conserved across all subtypes of Class D WHD proteins (Fig. 1B), it is not absolutely required by them; e.g., it is absent in the repressing WHD protein Genesis, the murine ortholog of avian CWH3 (compare Fig. 1B to the sequence in Sutton *et al.*, 1996). The more likely candidate for the major repressing moiety is the qualitatively conserved P/A/Q-rich region between the WHD and region II, which is necessary and sufficient for mimicking the gene-repressing activity of full-length CWH3 (Freyaldenhoven *et al.*, 1997b). Higher-resolution deletion assays will be needed to confirm this hypothesis.

The Role of foxD5a in Dorsal Axis Formation

Several experiments indicate that *foxD5a* has a role in dorsal axis formation. Its maternal expression is in the axis-precursor cells (Bauer *et al.*, 1994) and its zygotic expression is in the neural ectodermal portion of the Organizer. Its expression in animal caps and whole embryos initiates axis formation, and induces several mesodermal and neural markers. However, *foxD5a* does not initiate all

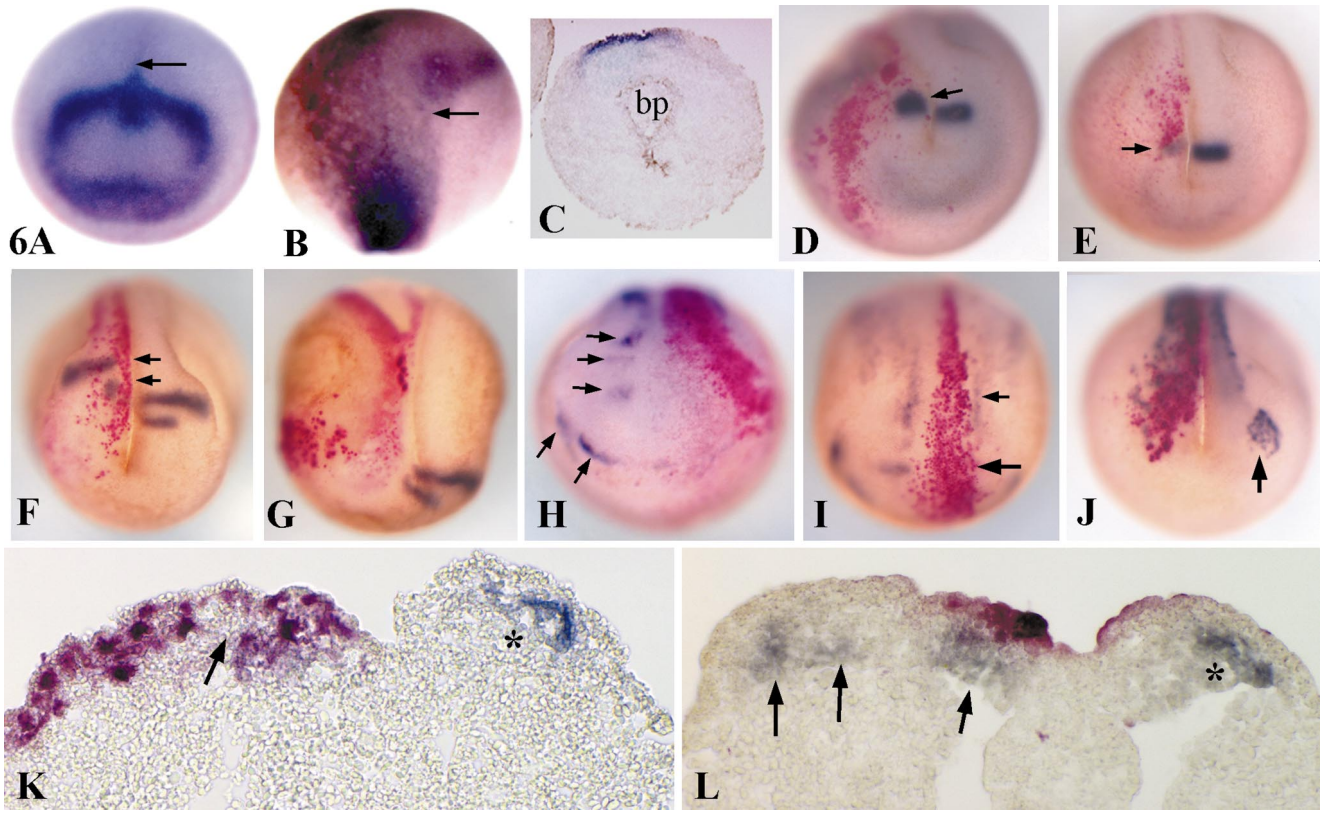


FIG. 6. Expression of exogenous *foxD5a* in the neural plate expands anterior markers and represses differentiation markers. (A) Anterior view of a control embryo (stage 14) stained for *otx2* expression (blue). Staining encircles the anterior neural plate and does not extend into the hindbrain/spinal cord domain (arrow). (B) Dorsal view of a *foxD5a* mRNA-injected embryo (stage 14) stained for *otx2* expression. Arrow denotes comparable level of arrow in A. Otx2 expression expands posteriorly (toward the bottom). (C) Transverse section of embryo similar to that in B, demonstrating ectopic *otx2* expression (blue) in the neural ectoderm at the level of the blastopore (bp). (D) Anterior view of stage 16 embryo stained for en2 expression (blue). On the side of *foxD5a* injection (red) the *en2* stripe (arrow) is smaller and slightly more posterior than the control stripe (right side). (E) Anterior view of stage 18 embryo. *en2* expression (blue) is repressed (arrow) in the cells expressing injected *foxD5a* mRNA (red). (F) Anterior view of stage 18 embryo. On the side of *foxD5a* injection (red) the *Krox20* stripes (blue) are more posterior (arrows) than the control stripes (right side). The rhombomere 3 stripe also is significantly smaller. (G) Dorsal view of stage 20 embryo. *Krox 20* expression is completely repressed on the side of *foxD5a* injection (red). Posterior is to the top of the figure. (H) Anterior view of stage 15 embryo. *Xngnr1* expression (blue) on the control side (left) is expressed in five patches (arrows). These patches are repressed in cells expressing injected *foxD5a* mRNA (red). (I) Dorsal view of stage 15 embryo. One horizontal stripe (large arrow) of *Xngnr1*-expressing cells (blue) is missing and one longitudinal stripe (small arrow) is smaller on the side of injected *foxD5a*-expressing cells (red). Posterior is to the top of the figure. (J) Anteriodorsal view of stage 18 embryo stained for *n-tubulin* expression (blue). The anterior patch (arrow) of the trigeminal ganglion is repressed on the side of injected *foxD5a*-expressing cells (red). (K) Transverse section from the spinomedullary level of stage 16/17 embryo demonstrating an area (arrow) in which *n-tubulin* expression (blue) is repressed on the side of injected *foxD5a* expression (red). Asterisk denotes a cluster of *n-tubulin*-expressing cells on the control side. (L) Transverse section from the caudal spinal level of stage 19/20 embryo demonstrating expansion (arrows) of clusters of *n-tubulin*-expressing cells (blue) on the side of injected *foxD5a* (red). Asterisk denotes a cluster of *n-tubulin*-expressing cells on the control side.

the elucidated steps in dorsal axis formation, indicating that it is a downstream component in this developmental pathway. First, *foxD5a* is not likely to be a component of the mesoderm inductive step, even though it upregulates some mesoderm markers. Although *foxD5a* expression is early (late stage 8), it is confined to the noninvoluting dorsal marginal zone; paraxial mesoderm expression is very low during late gastrulation and reaches moderate to high levels only during secondary gastrulation in the tail bud. Second, *foxD5a*-induced secondary axes are not complete and early hallmark genes of mesoderm induction (Xbra1, Xbra3, eFGF, chordin) are not induced. Nonetheless, the elongation of *foxD5a*-injected animal caps is consistent with a recent proposed function for the *XFD-12′* allele in controlling convergent-extension movements of the presumptive neural ectoderm (Fetka *et al.*, 2000). These results indicate

that *foxD5a* does not participate in the mesoderm inductive pathways of the primary axis, but may very well do so in the tail bud. The upregulation of somitic markers (m-actin, 12/101 antigen) in explants and ventral lineages may result from the recapitulation of a later *foxD5a*-mediated program in the paraxial mesoderm of the tail bud.

A second potential role for *foxD5a* in axis formation is in the establishment of the neural ectoderm. This gene is highly expressed in the presumptive neural ectoderm at the earliest stages in which the tissue can be identified, and it can upregulate at least one neural inducing gene (*Xnr3*) and several neural markers. In fact, its phenotypes are very similar to those described for Xnr3 (Hansen *et al.*, 1997), a gene that shares a similar expression domain and is induced by *foxD5a* in animal caps. Its expression domain would indicate that *foxD5a* is activated either by signaling from the Nieuwkoop Center or by neural-inducing molecules. We demonstrate that *foxD5a* is the target of the former because it can be readily induced in animal caps and in ventral vegetal sites by injection of *siamois* mRNA. Given that *siamois* expression extends into the future Organizer region (Lemaire *et al.*, 1995; Ding *et al.*, 1998), and that *foxD5a* expression is observed as early as stage 8, it is conceivable that *foxD5a* is a direct target of *siamois*. However, because *siamois* activates a number of Organizer transcription factors and signaling molecules (Carnac *et al.*, 1996; Lemaire and Kodjabachian, 1996; Kessler, 1997), this must be experimentally determined.

It is significant that *foxD5a* is not robustly induced in animal caps by the BMP antagonists noggin and chordin, whereas there is moderate induction by cerberus. In the intact embryo, noggin- and chordin-expressing mesoderm is adjacent to *foxD5a*-expressing neural ectoderm (Fig. 2H), whereas cerberus-expressing endoderm is slightly more distant (Fetka *et al.*, 2000). In light of cerberus's antagonism of Wnt and nodal-related proteins in addition to BMPs (Piccolo *et al.*, 1999), it is likely that the induction of *foxD5a* requires more than simple BMP antagonism. Although independent antagonism of Wnt or nodal-related proteins with two different cerberus-deletion constructs reduced the expression of the *XFD-12′* allele, it was not eliminated (Fetka *et al.*, 2000). Conversely, neither Wnt8 (Fig. 4D) nor Xnr1 (Fetka *et al.*, 2000) significantly induces *foxD5a* in animal caps. These results suggest that a complex of factors downstream of *siamois* optimally regulate *foxD5a* transcription. In agreement with this idea, Wnt8 and noggin (and chordin; Solter *et al.*, 1999; Fetka *et al.*, 2000) can induce *foxD5a* (or *XFD-12′*) in the ventral marginal zone of whole embryos, indicating that there are factors in this region, which are missing from animal caps, that facilitate the induction of *foxD5a*. One candidate is FGF, which maintains the expression of the *XFD-12′* allele in the gastrula (Solter *et al.*, 1999; Fetka *et al.*, 2000). Together these results demonstrate that *siamois*-mediated signaling is the likely primary initiator of *foxD5a* transcription, and that this is not effected simply by the antagonism of BMPs. However, the details of what components downstream of *siamois* directly regulate *foxD5a* await elucidation.

foxD5a Function in Establishing the Neural Ectoderm

foxD5a is expressed throughout the presumptive neural ectoderm, upregulating one neural inducer and several neural markers, and its expression on the dorsal side expands the neural plate. These results are all hallmarks of a molecule that is involved in neural induction. However, when ectopically expressed, *foxD5a* does not induce ectopic neural markers. Neither does it expand the dorsal mesoderm, which could lead to a broader domain of neural ectodermal cells. Therefore, *foxD5a* most likely influences cells that have already responded to neural induction, and are committed to neural fate but have not yet differentiated. This conclusion is supported by the lack of a FoxD5a effect on *geminin*, a neural ectoderm gene proposed to be expressed as an early response to neural induction (Kroll *et al.*, 1998). One candidate mechanism for neural plate expansion by FoxD5a would be the control of proliferation. For example, geminin, which also expands the neural ectoderm, is structurally related to proteins that regulate progression through the cell cycle (Kroll *et al.*, 1998). Indeed, several Fox proteins were previously posited to play roles in cell proliferation (reviewed in Vogt *et al.*, 1997). One of these, *Xlens1*, causes hypertrophy of the presumptive lens ectoderm (Kenyon *et al.*, 1999). However, repression of proliferation by antimitosis drugs did not eliminate the *foxD5a* neural plate phenotype nor did *foxD5a*-expressing cells express a proliferation marker (PCNA) at a higher frequency than did surrounding cells. We thereby conclude that *foxD5a* does not cause expansion of the neural plate by increasing the rate of cell division.

Another mechanism whereby a precursor population could be increased is by preventing differentiation. By maintaining a precursor pool in an immature state, cells can continue to proliferate at the status quo rate even in the presence of differentiation factors. For example, activation of Notch prevents cells from committing to a particular fate, thereby controlling the timing at which cells differentiate (Chitnis, 1995). Several observations indicate that repression of differentiation is the likely mechanism of the effects of *foxD5a* on the neural plate. The more posterior expression of forebrain, midbrain, and hindbrain marker genes may be the result of the neural plate maintaining its initial anterior state (Gould and Grainger, 1997; Nieuwkoop, 1997). The repression of the later-expressed position-specifying (*en2, Krox20*), proneural (*Xngnr1, NeuroD*), and neural differentiation (*n-tubulin*) genes in the neural plate, and the expansion of differentiated neurons at neural tube stages all indicate a delay in the differentiation process. In analogy to Notch activation, this indicates an increase in the precursor pool, which leads to a later increase in the number of differentiated cells. In fact, the expansion of the mesodermal and epidermal tissues in the partial secondary

axes after ventral *foxD5a* mRNA injections also may be accounted for by the delayed differentiation of their precursors. These data indicate that the normal role of *foxD5a* in the presumptive neural ectoderm is to repress genes that induce a cell to begin to differentiate, thereby establishing and/or maintaining the immature neural plate.

Several *fox* genes are expressed in the *Xenopus* neural plate, raising the question of whether their functions are redundant. We propose that they are not. First, unlike *foxD5a*, most *fox* genes in the neural plate are induced strongly by BMP4 antagonists. Second, their domains of expression only partially overlap. *XFD1/XFKH1/pintallavis* and *XFKH5* are expressed in the floor plate (Dirksen and Jamrich, 1992, 1995; Knöchel *et al.*, 1992; Ruiz i Altaba and Jessell, 1992), *XFKH4*/*XBF1* and *XBF2* are expressed predominantly in the anterior neural plate (Dirksen and Jamrich, 1995; Bourguignon *et al.*, 1998; Mariani and Harland, 1998), and *XFKH6* is expressed along the lateral border of the midbrain/hindbrain region (Lef *et al.*, 1994; Dirksen and Jamrich, 1995; Scheucher *et al.*, 1995). In contrast, *foxD5a* is expressed broadly throughout most of the neural plate, but only for a limited time. Third, these Fox proteins represent several classes (XFD1 = A; XFKH5 = B; XFKH4/XBF1 = G; XBF2, XFKH6, and FoxD5a = D), suggesting that they would have different functions. Finally, although analyses of gene function have been reported for only a few of these genes, those studied are not identical. *XFD1* represses anterior neural structures and expands posterior neural tissue (Ruiz i Altaba and Jessell, 1992). *XBF1*, *XBF2*, and *foxD5a* all expand the neural plate and repress differentiation. But even among these three genes there are distinctions beyond their different expression domains. Whereas *foxD5a* expands an anterior character (*otx2*) in the neural plate and represses more posterior markers (*en2*, *Krox20*), *XBF2* induces both anterior and posterior neural markers. *foxD5a* does not directly convert ectoderm to a neural fate, whereas *XBF2* does. *foxD5a* represses differentiation, whereas *XBF1* is posited to act in the early steps of neuronal differentiation. Subtle variations in function of these several *fox* family members in the neural ectoderm may regulate discrete processes, leading from the acquisition of neural competence to full neural differentiation. The elucidation of these processes and the role of Fox proteins in them will be key to understanding the translation of neural inductive signaling into the mature patterning and differentiation of the nervous system.

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